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Platelet-mediated clumping of *Plasmodium falciparum* infected erythrocytes is associated with high parasitemia but not severe clinical manifestations of malaria in African children

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Abstract

Platelet-mediated clumping of *Plasmodium falciparum* infected erythrocytes is an adhesive phenotype commonly found in field isolates that has previously been associated with severe malaria. Here, clumping was assessed in 131 isolates from Malian children. The clumping phenotype was seen in 6% (n=51) of uncomplicated malaria, 24% (n=51) of severe malaria, and 45% (n=29) of high parasitemia non-severe malaria isolates. Multivariate analysis indicated that clumping was strongly positively associated with parasitemia ($F_{1,122}=24.1$, $p<0.001$) but not with disease category ($F_{2,122}=1.8$, $p=0.17$). Therefore platelet-mediated clumping in Malian *P. falciparum* isolates is primarily associated with high parasitemia and not with severe clinical manifestations of malaria.

Introduction

Plasmodium falciparum, the most virulent of the parasites causing human malaria, has the unique ability to sequester in the vasculature of diverse host organs.¹ Sequestration resulting from the adherence of erythrocytes that contain mature forms of *P. falciparum* (trophozoites and schizonts) to the vascular endothelium occurs in all infections. However, several adhesive phenotypes have been associated with severe pathological outcomes of malaria, such as the formation of rosettes, and the sequestration of infected erythrocytes (IEs) in the microvasculature of the brain and the placenta.¹ Recently, it has been shown that some *P. falciparum* IEs can bind platelets to form platelet-mediated clumps², a cytoadherence

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phenomenon that has been associated with severe malaria in Kenya² and Thailand³. Although *P. falciparum* seems to use CD36 on the surface of platelets as a receptor for platelet-mediated clumping of IEs², nothing is known about the parasite proteins involved in this cell-to-cell interaction. Here, we investigated the association between disease severity and the platelet-mediated clumping phenotype of *P. falciparum* isolates from Malian children.

Methods

Study site and field isolates

Parasite isolates were collected in Bandiagara, Mali, an area with intense seasonal transmission of *P. falciparum* (up to 20-60 infected bites per person per month at the peak of the July-December transmission season).⁴ The samples were collected as part of the Bandiagara Malaria Project case-control study in which severe malaria cases were matched by age, residence and ethnicity to uncomplicated malaria controls.⁵ Blood samples were collected from children with malaria after informed consent from parents or guardians, and all protocols received institutional review board approval. The WHO criteria for severe malaria were applied⁶, although patients with hyperparasitemia (>500,000 parasites per microlitre of blood) and no other symptoms or signs of severe disease were analysed as a separate group. Previous studies indicate an excellent prognosis for children with non-severe hyperparasitemia, and this category can therefore be considered as a form of uncomplicated malaria with particularly high parasite densities.⁵ Uncomplicated malaria cases were children with *P. falciparum* infection and fever but with no symptoms or signs of severe malaria and no hyperparasitemia.

Parasite culture

Blood samples were depleted of lymphocytes via density centrifugation and then were suspended in Glycerolyte and frozen to -70°C . Frozen samples were shipped to Edinburgh where they were thawed by standard methods. Briefly, the isolates were diluted in a gradient of salt solutions and washed in RPMI 1640 medium containing 2mM glutamine, 25mM Hepes, 20 mM glucose and 25 $\mu\text{g}/\text{ml}$ gentamicin (incomplete RPMI) before culturing in complete RPMI (incomplete RPMI supplemented with 10% human AB serum). The parasites were cultured in 3% CO_2 , 1% O_2 , 96% N_2 at 37°C . Cultures were monitored by Giemsa-stained thin smears for 18-36 hours, and only those with normal morphology that matured to the pigmented-trophozoite stage were included in the study.

Clumping assays

Clumping was assessed when the parasites reached the mature pigmented trophozoite stage, using methods described previously³ with minor modifications (described below). Briefly, parasite cultures were suspended at 2% haematocrit in 10% platelet-rich plasma (PRP) from an AB+ malaria-naïve donor (to avoid ABO compatibility problems) in incomplete RPMI medium (final concentration 1×10^7 platelets per ml). 25 $\mu\text{g}/\text{ml}$ of ethidium bromide was added and the mixture was gently rotated for 30 minutes at room temperature. A wet preparation was viewed on a fluorescence microscope and 500 infected red cells were counted and scored for clumping, with 3 or more IEs adherent to each other constituting a clump. The clumping frequency is expressed as the percentage of IEs in clumps out of 500 IEs counted. An aliquot of each culture was also set up with 10% platelet-poor plasma (PPP) as described previously³, and clumping assessed as above, however no clumping was seen in any sample in PPP.

Rosetting assays

Rosette frequency was assessed by staining an aliquot of culture suspension with 25 µg/ml of ethidium bromide. A wet preparation of the suspension (2% haematocrit) was viewed with a fluorescence microscope and the number of mature-IEs binding 2 or more uninfected erythrocytes was counted. The rosette frequency is the percentage of IEs in rosettes out of 200 IEs counted.

Statistical analysis

Univariate analysis was carried out using Statview (version 5, SAS Institute, Inc.). Multivariate analysis was carried out using S-PLUS 6.0 (Release 1, Insightful Corp.), using Generalized Linear Models (GLM). Since the response variables were proportions, and therefore bound between 0 and 1, they were analysed using binomial errors with a logit linear predictor.^{7,8} The percentages of infected erythrocytes forming clumps were analysed as counts with binomial errors. Explanatory variables in the statistical model included blood group, category of disease (severe, hyperparasitemia, and uncomplicated - as defined above), % parasitemia, % rosetting, age and haemoglobin level. Models were fitted as follows. All explanatory terms were fitted including interactions up to second order where possible. Interactions including more than two terms (e.g. a third order interaction between three explanatory terms) were not permitted due to small sample sizes. This would have reduced residual degrees of freedom, making the model unstable, and would also have increased the risk of incurring both type I and type II errors.^{7, 8} The statistical significance of a term in a GLM with binomial errors was assessed by the change in deviance of the model when the term was dropped from it. To correct for overdispersion, significance of a term was assessed using an F ratio. Minimal models were obtained by step-wise deletion of non-significant terms, using an α -value of 0.05.

Results

Clumping was assessed in 131 *P. falciparum* isolates from Malian children (51 from children with uncomplicated malaria, 29 from children with non-severe hyperparasitemia and 51 from children with severe malaria). The characteristics of the patients from which the parasite isolates were derived are summarised in Table 1. The children in the three disease categories did not differ significantly in age, however, the children with severe malaria had lower haemoglobin levels than the other two groups and the children with uncomplicated malaria had lower parasitemia levels than the other two groups (Table 1). The severe malaria category comprised 16 children with unrousable coma (Blantyre coma score = 2), 12 non-comatose children with impaired consciousness or prostration, 14 children with repeated seizures, 5 children with severe malarial anaemia (Hb <5 g/dl) and 4 children with assorted other severe malaria syndromes (jaundice, haematuria, renal failure). 18 of the severe malaria children had concomitant hyperparasitemia.

The clumping phenotype was seen in 28/131 (21.4%) of all isolates and there were significant differences between clinical categories in the number of isolates showing clumping. 3/51 (6%) of the uncomplicated malaria isolates formed clumps, 12/51 (24%) of severe malaria isolates formed clumps and 13/29 (45%) of the non-severe hyperparasitemia isolates formed clumps ($\chi^2=16.9$, 2df, $p<0.0001$). The distribution of clumping frequencies in the three disease categories is shown in Figure 1. Higher levels of clumping were seen in the non-severe hyperparasitemia and severe malaria categories than the uncomplicated malaria category (Kruskal Wallis test, $p<0.001$). Multivariate analysis indicated that clumping was strongly positively associated with parasitemia ($F_{1,122} = 24.1$, $p<0.001$). The apparent association between clumping and disease category shown above was directly due to differences in the parasitemia in each category. Thus when parasitemia was included in

the statistical model, disease category was a non-significant term ($F_{2,122} = 1.8$, $p=0.17$). Patient age and haemoglobin were also non-significant terms in the model. Clumping was, however, found to be negatively associated with rosette frequency ($F_{1,122} = 5.8$, $p<0.001$). Rosette frequency itself was strongly associated with severe disease in this sample set (uncomplicated malaria: median rosette frequency (RF) 3%, interquartile range (IQR) 0-15.0, non-severe hyperparasitemia: median RF 7%, IQR 2-16.8, severe malaria: median RF 20%, IQR 10-36.3, Kruskal Wallis test $p<0.0001$), as has been observed previously in isolates from African children.^{9,10} The analysis of factors affecting clumping was repeated using a smaller dataset consisting only of severe disease cases, so that the effects of sub-category of severe disease could be considered. The main findings were very similar, however there were no significant differences in clumping between the subcategories of severe disease ($F_{4,43} = 1.08$, $p=0.38$).

Discussion

The main finding of this study was that *P. falciparum* platelet-mediated clumping is not associated with severe clinical manifestations of malaria, but is significantly associated with high parasitemia, a result that differs from those reported previously from Kenya² and Thailand.³ The previous studies compared clumping in isolates from severe malaria cases with that in isolates from uncomplicated malaria controls that had significantly lower parasite burdens than the severe malaria cases.^{2,3} On multivariate analysis, the previous studies found both high parasitemia and severe disease (cerebral malaria in Thai adults) to be independently associated with clumping.^{2,3} In the present study, we were able to extend the investigation of the effects of parasitemia and severe disease on clumping by including a high parasitemia non-severe malaria control group. Our results indicate that parasitemia but not disease category is the most significant factor in relation to clumping in *P. falciparum* isolates from African children. These results highlight the fact that inclusion of non-severe controls with high parasite burdens equivalent to those in severe cases might be of great importance, not only for the study of clumping, but also in the investigation of other parasite phenotypes associated with severe malaria in Africa.^{11,12,13} Non-severe hyperparasitemia is a feature of malaria in children in semi-immune populations such as those found in sub-Saharan Africa, however in low transmission areas such as Thailand, such non-severe hyperparasitaemic patients are rare and high parasitemia is highly predictive of severe disease.¹⁴

One limitation of the study reported here is that, as in the previous Kenyan study², the analysis was carried out primarily with a mixed group of severe malaria syndromes. Larger studies are needed to examine the association of clumping with specific sub-types of severe disease, as done in Thailand where clumping was associated with cerebral malaria but not with the multi-organ failure type of severe malaria.³ In the present study, clumping in isolates from cerebral malaria cases (unrousable coma, $n=16$), did not differ significantly from clumping in isolates from patients with other forms of severe disease ($n=35$). The lower absolute numbers of platelet-mediated clumps found in our study are probably explained by methodological differences (notably, in haematocrit and time of the reaction) between this and the study carried out in Kenya², although our methods are comparable to those used in Thailand.³

Another possible limitation of the current study is that the isolates examined were all frozen immediately after collection, and later thawed and cultured for 18-36 hours *in vitro* before clumping was assessed. Previous work has shown that it is possible for *P. falciparum* isolates to show different antigenic profiles after being frozen and thawed, however, the majority of isolates show very similar antigenic profiles before and after freezing.¹⁵ In addition, in a small study of twelve field isolates we found that rosette frequency, a

virulence-associated phenotype^{9,16} that is mediated by the variant surface antigen PfEMP1,¹⁷ is not affected by freezing. There was a very strong positive correlation between the rosette frequency of fresh isolates compared to the rosette frequency of the same isolates after freezing and thawing, $r^2=0.91$, $P<0.001$, least squares regression, $n=12$). We therefore think it is unlikely that the use of frozen isolates had a major impact on the results of this study.

It is unclear whether the relationship between clumping and parasitemia shown here represents a genuine biological phenomenon (ie isolates that grow to high parasitemia in their human hosts do have higher clumping frequencies than those that only reach low parasitemia) or whether it is merely a technical artefact of the assay (ie clumping is seen more readily when the parasitemia of the culture is high). Preliminary experiments with *P. falciparum* laboratory strains suggest that the latter is the most likely explanation (M. Arman unpublished data). In future studies of clumping and disease severity it may be prudent to control for the effect of parasitemia, either by using parasitemia-matched severe malaria cases and uncomplicated malaria controls, or by diluting all isolates to a standard starting parasitemia before performing the clumping assay.

Independently of the platelet-mediated clumping phenotype, there is evidence suggesting that platelets may have an important role in the pathology of malaria. Platelet sequestration has been described in the cerebral vessels of children who have died with severe malaria.¹⁸ Moreover, platelets have been involved in brain endothelial alterations induced by *P. falciparum* in *in vitro* models.¹⁹ The fact that platelet-mediated clumping was previously associated with severe malaria^{2,3} and the large size of the clumps found *in vitro* have led to the hypothesis that platelet-mediated clumps of *P. falciparum* IEs may contribute to disease severity by increasing vascular resistance and causing local disturbance of blood flow. Nevertheless, the association of clumping with high parasitemia but not with severe malaria shown in the present study suggests that the interaction of IEs with platelets in the context of clumping may not be a causative agent of severe disease. It will be important in future to try and understand the way in which *P. falciparum* parasites are affected by the formation of platelet-mediated clumps. In this regard, it is of interest to note that some studies have demonstrated that platelets are able to inhibit the growth and maturation of *P. falciparum* infected erythrocytes *in vitro*.^{20,21}

In summary, the data from this study challenge the conclusions of previous work by indicating the platelet-mediated clumping is associated with high parasitemia but not with severe clinical manifestations of malaria. Further work is needed to examine the relationships between clumping, parasitemia and disease severity in other areas, and to understand the physiological effects of platelet-mediated clumping on *P. falciparum*.

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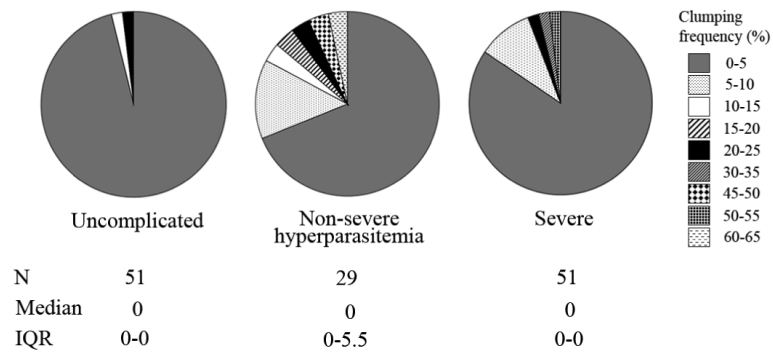
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**Fig 1.**

The distribution of clumping frequencies in *P. falciparum* isolates from Malian children with uncomplicated malaria, non-severe hyperparasitemia and severe malaria. The clumping frequency is the percentage of mature pigmented-trophozoite infected cells in clumps of 3 or more infected cells, out of 500 infected cells counted. The differences in clumping between disease categories are statistically significant ($P < 0.001$, Kruskal Wallis Test). IQR: interquartile range.

Table 1

Summary of patient characteristics

Disease category	N	Age ^a (months)	Hb ^a (g/dl)	Pt ^{a,b} (%)
Uncomplicated malaria	51	41.2 (27.4)	9.8 (2.5)	2.4 (1.5)
Non-severe hyperparasitemia	29	47.1 (28.1)	10.0 (2.3)	11.5 (6.2)
Severe malaria	51	34.8 (23.7)	8.2 (1.8)	7.3 (5.9)
P value ^c		0.12	0.0003	<0.0001

^a Mean (standard deviation)^b Pt: parasitemia^c ANOVA